The University of Chicago

STUDIES ON THE TRYPSINOGEN, ENTEROKINASE, AND TRYPSIN SYSTEM

ASSAY METHODS FOR TRYPSINOGEN AND ENTEROKINASE

A PART OF A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY AND PHARMACOLOGY
1931

By
ROBERT WESLEY BATES

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Reprinted from
The Journal of Biological Chemistry
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ASSAY METHODS FOR TRYPSINOGEN AND ENTEROKINASE

By ROBERT W. BATES

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(Received for publication, April 30, 1935)

Studies by Helmer (1) have not confirmed the addition compound theory for the mode of activation of trypsinogen by enterokinase recently revived by Willstätter, Waldschmidt-Leitz (2), and coworkers. He did not, however, conclusively prove the catalytic theory instead. In this paper we present conclusive evidence in favor of the catalytic theory.

The reactions below may be considered to represent these two theories and the action of trypsin on proteins, respectively.

Trypsinogen (T) + enterokinase (K) =
trypsin (A) + enterokinase (K) + ? (1)

Trypsinogen + enterokinase $\rightleftharpoons$
trypsinogen-enterokinase complex (trypsin) (2)

Protein + trypsin = digestion products + trypsin (3)

Procedure

Enterokinase—Fresh or desiccated hog duodenal mucous membrane was extracted at pH 8.0 and centrifuged. K_1 was the acetone-dried precipitate obtained at about pH 5.4. K_3 was the acetone-dried precipitate obtained by adding 2 to 5 volumes of acetone to the soluble fractions at pH 5.4. K_3 was 4 to 8 times as potent per mg. as K_1.

Trypsin-Free Trypsinogen—Perfectly fresh finely ground hog pancreas was suspended in an equal weight of water and concentrated HCl immediately added to obtain a mixture at pH 1.8. This was allowed to stand for 3 hours at 37°. (The enterokinase,

prokinase, or other activating substances are completely destroyed by this treatment without much, if any, trypsinogen being destroyed) (see Table III and Fig. 4).) After filtering through cheesecloth, the filtrate was adjusted with NaOH to the point of maximum flocculation, usually about pH 6.0. The resulting precipitate was removed by centrifuging and acetone added. A slight precipitate at 45 per cent acetone concentration was discarded and acetone added to a concentration of 75 per cent by volume. The resulting precipitate, referred to as T_7, was dried with acetone; yield, 0.8 per cent. The potency was 0.045 mg. = 1 unit, of which less than 0.1 per cent was trypsin (see Table I). T_2 was similarly prepared by acetone precipitation.

Trypsin—An aqueous extract of fresh hog pancreas, after standing at pH 4 to 5 for 24 hours at room temperature, was precipitated with 3 volumes of acetone and the precipitate dried. Complete activation to trypsin had occurred. This precipitate, A_1, had a potency of 0.025 mg. = 1 unit.

Assay Method—The assay method was essentially that of Helmer (1). An 8 per cent solution of casein (Merck's Hammarsten grade) in 0.54 per cent Na_2CO_3 was used as substrate. 75 cc. of the substrate at 37.5° were added to 25 cc. of the enzyme solution, also at 37.5°, making a total volume of 100 cc. All digestions and all activations were carried out in a constant temperature room at 37.5° ± 0.5°.

At the beginning and at the end of the 4 hour digestion period, 25 cc. samples of the digestion mixtures were pipetted into 3.5 cc. of 2 N acetic acid with vigorous stirring during the addition. These samples were then placed in a constant temperature room at 25° for an hour before filtering through hard, quantitative filter paper.

All readings were made with a Zeiss dipping refractometer at 25° ± 1° and are recorded in terms of scale divisions. The control readings, from which the change in refractive index in scale divisions, R.I.Δ, was calculated by difference, were always taken at the same time as those of the digestion samples so that temperature corrections were not necessary.

Control digestions containing casein and water, casein and trypsinogen, and casein and enterokinase, all at the same pH, were made at intervals. The controls containing enterokinase never differed from those containing water.

Observations

Standard Curve for Trypsin Digestion—Schütz's law expresses the rate of digestion better than any other equation according to the work of Moelwyn-Hughes, Pace, and Lewis (3), Northrop (4), Willstätter, Waldschmidt-Leitz, Dunaiturria, and Künstner (5). Schütz's square root law, however, has been found not to hold in the early stage of digestion nor does it hold in the late stages of digestion.

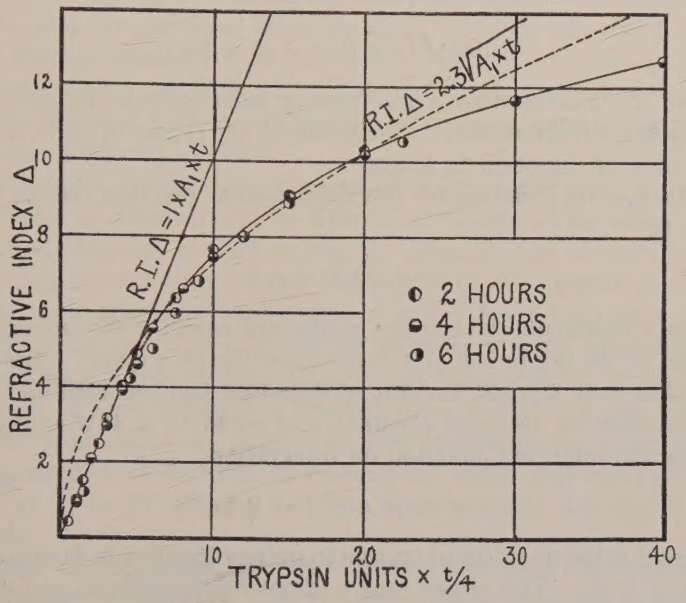

Fig. 1. The standard digestion curve is the smoothed curve drawn through the experimental points. The broken curve is the theoretical Schütz curve. The straight line is the theoretical line used for graphical conversion of actual digestion values into theoretically equivalent values. 1 trypsin unit = 0.025 mg. of A_1.

An experimental curve (Fig. 1) was therefore made with trypsin, A, for the graphical interpolation of the potency of any sample whether it fell within or without the range where Schütz's law holds. Samples of the digestion mixtures were removed at intervals of 2, 4, and 6 hours to show that the amount of digestion was directly proportional to the time of the digestion. The quantity of enzyme has been multiplied by (time)/4 so that the data at

all three times could be plotted on the standard 4 hour time basis against the change of refractive index in scale divisions. A smooth curve was drawn through the points obtained from the 4 hour samples. The fact that the points for the 2 hour digestion lie above and those for the 6 hour digestion below this curve suggests that the trypsin is gradually destroyed during digestion.

The ideal curve obtained from the Schütz equation is also plotted with 4 hours taken as unit time. The expression for this curve is

$$\text{R I.}\Delta/\sqrt{A_1 \times (t/4)} = k_s = 2.3 \tag{4}$$

where R.I.Δ is the change in refractive index in scale divisions on a dipping refractometer, A_1 the quantity of trypsin in units, and t the time of digestion in hours.

The general equation for the unimolecular reaction rate in this case is

$$dx/dt = k_1(c - x)A \tag{5}$$

where x represents the amount of digested casein, c the amount of casein at the beginning, and A the quantity of trypsin. Assuming that only a small amount of digestion has occurred so that $(c-x)$ may be assumed constant and equal to c, this equation yields the following equation on integration.

$$x = k_2 \times A \times t = \text{R.I.}\Delta \tag{6}$$

where A is the quantity of trypsin in units, t the time in hours, and $k_2 = c \times k_1$. This shows that a direct relationship should exist between the amount of digestion (R.I.Δ) and the quantity of enzyme, A_1, at a constant time of digestion, when the amount of digestion is small. That this direct relationship holds in this case below a R.I.Δ of 4.5 can be seen from the graph (Fig. 1).

Units of Trypsin and Trypsinogen—The proteolysis represented by a change of 1.0 scale division on a dipping refractometer under the empirical conditions here imposed was selected as a unit of proteolysis. A unit of trypsin is a quantity of trypsin that will cause this unit of proteolysis. A unit of trypsinogen is defined as a quantity of trypsinogen that will cause this unit of proteolysis when it is maximally activated.

Activation Studies

Solutions of trypsinogen and enterokinase were mixed at a noted time and kept at 37.5°. At various time intervals, 10 cc. samples of this mixture were added to a mixture of 75 cc. of casein substrate and 15 cc. of water. This digestion mixture was then shaken at 37.5° for 4 hours, when 25 cc. samples were precipitated as usual and the filtrate used for the determination of the refractive index. The 10 cc. samples of the mixed enzymes always contained 0.5 mg. of T_2 and the indicated amounts of K_1, or 0.1 mg. of K_1 and the indicated amounts of T_2. Unless salt was added, the enzyme solutions were practically salt-free. Control experiments with 0.5 mg. of T_2 with no kinase added, after 4 hours incubation followed by 4 hours digestion with casein, gave a maximum change of 0.2 scale division.

Activation of Constant Amount of Trypsinogen with Varying Amounts of Enterokinase in Absence of Casein—The data from these studies are plotted in Fig. 2. The amount of activation, which is directly proportional to the amount of digestion, is seen to be proportional to the amount of enterokinase from 0 time up to 30 minutes. The addition of more and more enterokinase speeds up the rate of activation so that the maximum amount of trypsin in each mixture is reached more quickly as one would expect from either the enzyme or addition compound theory. It is apparent that 0.25 mg. of K_1 does not completely activate 0.5 mg. of T_2 in 30 minutes but that approximately 1.0 mg. of K_1 would.

After the maximum activity is passed, the rapid destruction of the trypsin is apparent. No trypsin potency remained in any of the samples after 24 hours with the exception of the one containing 2.5 mg. of enterokinase. In this case a change of 0.5 scale division was obtained after 24 hours. Addition of enterokinase did not change this amount of digestion, but the addition of 0.5 mg. of T_2 and activation for 30 minutes gave a change of 5.8 scale divisions for 4 hours digestion, showing that the enterokinase has not been completely destroyed and that the loss of potency is due to the destruction of trypsin. There was no trypsin or trypsinogen (fresh kinase was added to one part) after 24 hours in the mixture containing 0.025 mg. of K_1 and 0.5 mg. of T_2. Trypsinogen alone would be only partially destroyed in 24 hours (see Table III), hence

all of the trypsinogen has probably been converted into trypsin. The greater stability of trypsin in the presence of excess enterokinase is perhaps due to the protective action of the contaminating inert protein. An analogous experiment with the addition of thymol gave almost identical data showing that the destruction is not due to bacterial action.

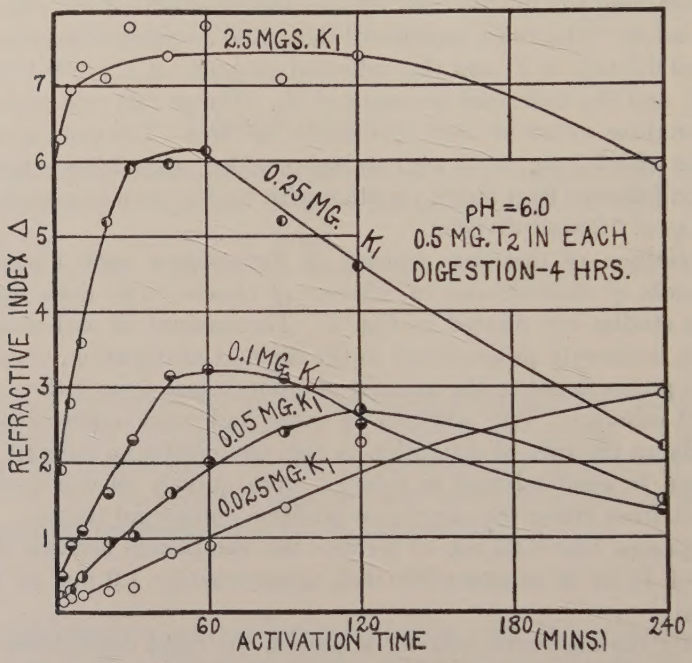

FIG. 2. The course of the activation of a constant quantity of trypsinogen with five different quantities of enterokinase.

Activation of Varying Amounts of Trypsinogen with Constant Amount of Enterokinase in Absence of Casein—The usual method of determining the trypsinogen content of any preparation is to add an excess of enterokinase in order to convert the trypsinogen completely and rapidly into trypsin, which can then be measured by a suitable method. If the addition compound theory is correct and the proteolytic enzyme is a trypsinogen-enterokinase complex (Equation 2), it should be possible to determine the enterokinase concentration of a preparation by adding an excess of

trypsinogen in order to convert all of the enterokinase into the compound trypsinogen-enterokinase. In other words, if one adds larger and larger amounts of trypsinogen to the same quantity of enterokinase, a point should be reached where all of the kinase is combined to form the hypothetical trypsinogen-enterokinase and the further addition of trypsinogen should cause no further increase in activity. Also the time to reach this maximum activation should decrease and would probably approach a value of 30 minutes or less as more and more trypsinogen is added if entero-

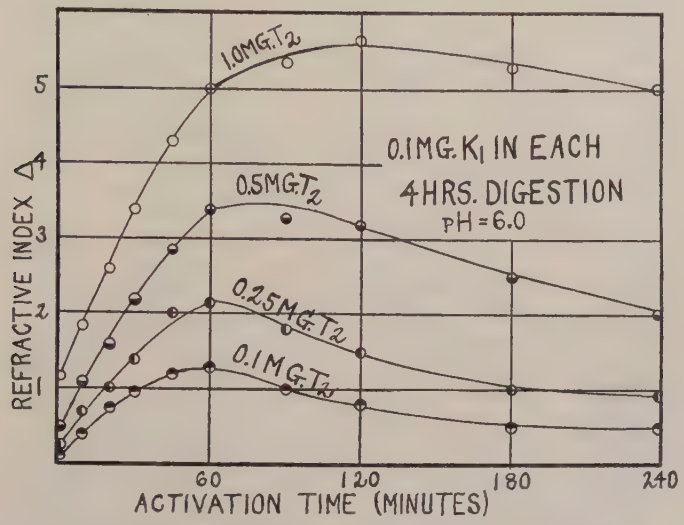

FIG. 3. The course of the activation of four different quantities of trypsinogen with a constant quantity of enterokinase.

kinase forms an addition compound with trypsinogen but not if enterokinase acts as an enzyme in the activation process.

That the addition compound theory does not hold is shown in Fig. 3. The quantity of enterokinase chosen was so small that even with the smallest quantity of T_2 (0.1 mg. = 2.2 units), the maximal activity was not reached. No maximal amount of activation of the enterokinase was obtained corresponding to the compound trypsinogen-enterokinase. However, as the quantity of trypsinogen was increased, the time to reach a maximal activity was also increased.

From Fig. 3 we see that 0.1 mg. of K_1 is able to produce the equivalent of 5 units of trypsin in the solution in 60 minutes. If 5.0 mg. or more of T_2 had been used, a still larger activity would be produced by 0.1 mg. of K_1 in 60 minutes, but it would have taken longer to produce the maximum activation than with less trypsinogen. These facts are incompatible with the addition compound theory but are what one would expect if enterokinase acts as an enzyme. The maximum activity obtained is a smaller

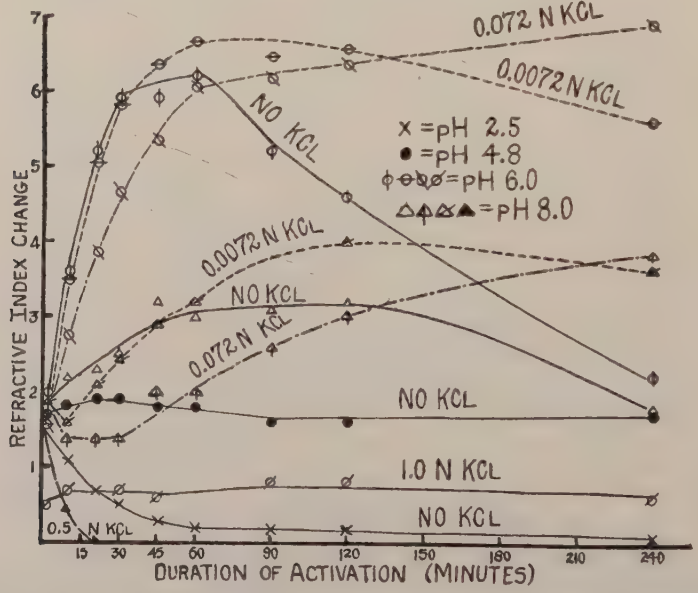

Fig. 4. Effect of pH and salt concentration upon the course of the activation of trypsinogen. All samples contained 0.5 mg. of T_2 and 0.25 mg. of K_1.

percentage of the total possible activity as the amount of trypsinogen is increased. This is what one would expect since it takes a longer time to reach the same percentage of the possible activity with the small proportion of enterokinase and as a result, more trypsin is destroyed in the meantime.

Effect of Salt Concentration and pH on Activation in Absence of Casein—Comparative studies at pH 2.5, 4.8, 6.0, and 8.0 clearly showed that the most rapid and complete activation was obtained at pH 6.0 (Fig. 4). Concentrations of 0.0072 N and 0.072 N

KCl at pH 6.0 did not affect the rate and extent of activation appreciably but did prevent or retard the destruction of trypsin. With N KCl at pH 6.0, no activation was observed. Similar experiments at pH 8.0 showed the same effects by 0.0072 and 0.072 N KCl but the extent of maximum activation was approximately only 60 per cent of that found at pH 6.0. At pH 8.0 in 0.5 N KCl, a rapid destruction of trypsin or trypsinogen or both was observed. At pH 4.8 and 2.5 no activation was observed, partly because the enterokinase was destroyed (see Table III).

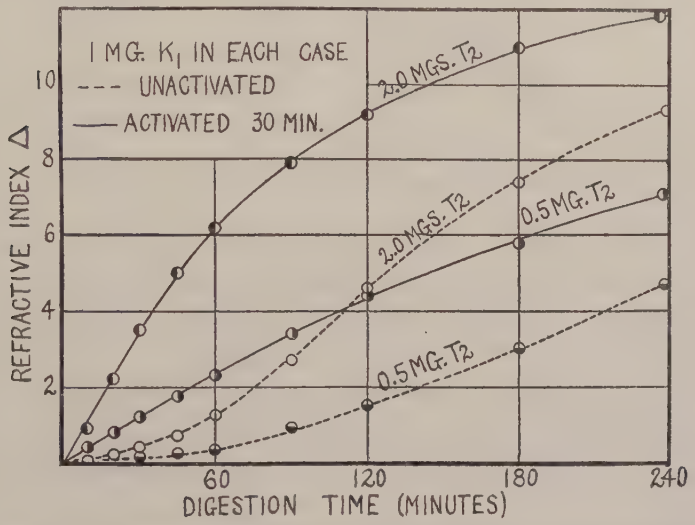

FIG. 5. The course of digestion by trypsinogen previously completely converted into trypsin and by the same quantity of trypsinogen and enterokinase added separately to the substrate at 0 time. The rate of digestion in the latter case increases with time; *i.e.*, the amount of trypsin is increasing.

Course of Activation in Presence of Casein—That trypsin is formed during incubation with the substrate is shown in Fig. 5 and in the 0 hour readings in Figs. 2 and 4. In the activated samples (Fig. 5), the trypsinogen and enterokinase mixtures were allowed to incubate for 40 minutes at pH 6.0. The casein was then added and samples removed for determination of refractive index at the time intervals noted. In the unactivated samples, trypsinogen and enterokinase were added directly to the casein substrate and the time of the addition of the last of the enzymes

was taken as the 0 time of digestion. Neither the trypsinogen nor the enterokinase alone caused any significant change in the refractive index readings.

In the previously activated samples, the rate of digestion was maximum at the beginning of the reaction. This rate was constant up to a change of 4.5 scale divisions, when it decreased to the extent indicated in the standard digestion curve, Fig. 1.

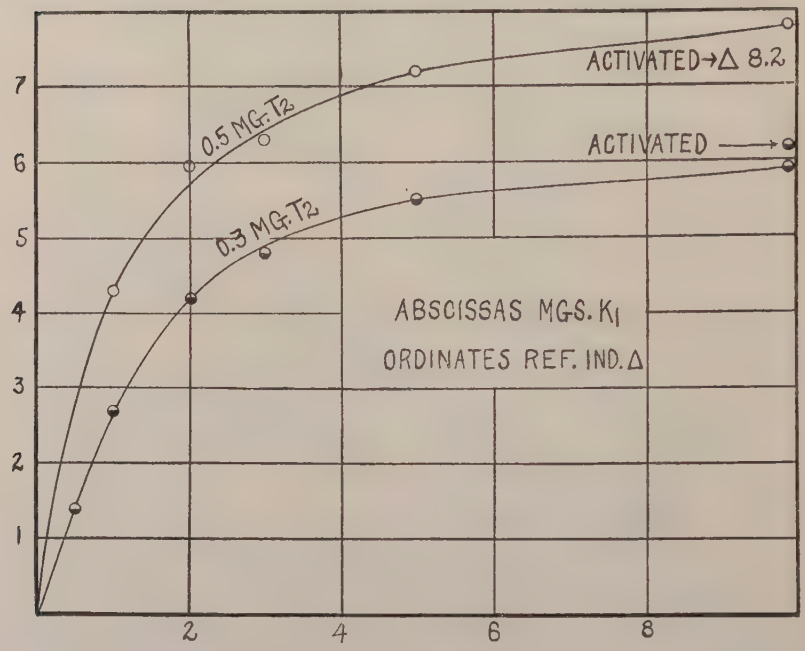

Fig. 6. The rate of activation of the trypsinogen in the presence of the substrate is increased by an excess of enterokinase so that the resulting amount of digestion is almost equal to that of an equivalent amount of trypsin.

On the other hand, the rate of digestion in the unactivated samples increased continuously as trypsin was formed. In the cases shown there was sufficient enterokinase to activate the trypsinogen completely in 2 to 3 hours so that the digestion curve thereafter was almost parallel to that of the previously activated sample.

Complete Activation in Presence of Casein—In Fig. 6, the results

are given from activating definite amounts of trypsinogen in the alkaline substrate with increasing concentrations of enterokinase. Thus 10 mg. of K_1 + 0.5 mg. of T_2 produced an amount of digestion which is equivalent to 0.9, the amount of trypsin obtained by starting with the same amount of completely activated trypsinogen. The results with the lower concentrations of K_1 indicate a direct relation between these concentrations and the amount of digestion. This suggests that a similar system, but with an excess of trypsinogen instead, could be used as a means of assaying enterokinase (6). These curves are strikingly similar to those given by Linderstrøm-Lang and Steenberg (7) with activation in the absence of the substrate in the assay of enterokinase.

That one can almost completely activate the trypsinogen in the presence of the substrate suggests a simple and fairly accurate method of assaying trypsinogen. This trypsinogen method is of value where we have present a large quantity of any substance, which would inhibit activation if carried out in the usual way; *e.g.*, high salt concentration, alcohol, glycerol, etc. In such cases a 5 cc. sample of the enzyme in 100 cc. of a digestion mixture would have this inhibiting substance diluted 20 times. This method was used in the stability experiments given in Table III, because of the difficulties of adjusting these solutions of varying acidities and alkalinities to the same pH and salt concentration for activation. The casein substrate is sufficiently buffered so that the small quantities of acid or alkali in the enzyme solutions would not alter the amount of digestion.

Activation in Presence of Substrate As a Function of Time and Concentration of Enterokinase and Trypsinogen. Theoretical—When one mixes the trypsinogen, enterokinase, and casein without previously allowing the trypsinogen and enterokinase to incubate together, the amount of digestion of the casein is a result of the two reactions (Equations 1 and 3) which occur simultaneously, the second depending upon the first.

The rate of the digestion of casein has already been considered and an equation established for it (Equation 6). Assuming the action of enterokinase to be that of an enzyme, an equation may be written for the rate of conversion of trypsinogen into trypsin.

$$dA/dt = k_3(T - A)K \qquad (7)$$

If A is assumed to be small with respect to T, so that $(T - A)$ can be considered equal to T, integration will give

$$A = k_3 \times K \times T \times t \tag{8}$$

Combining Equations 6 and 8 so as to eliminate A, one obtains the following.

$$\text{R.I.}\Delta = k_4 \times K \times T \times t^2 \tag{9}$$

This equation indicates that at the beginning of the reaction, the amount of casein digested is directly proportional to the amount of enterokinase and trypsinogen present and to the square of the duration of the reaction. Equation 9 would not apply if either the activation of trypsinogen or the digestion of casein were instantaneous or if one reaction occurred at a very rapid rate with respect to the other. Also T must be present in excess as we have assumed. Fig. 4 shows that at pH 8.0 and ionic strength of 0.072, which are of the same orders as in the casein substrate, the rate of activation is greatly retarded relative to the rate at pH 6.0. It is this slow rate of activation in the presence of substrate that makes this simple theoretical treatment applicable.

EXPERIMENTAL

Tables I and II contain experimental proof of the equations given above. The procedure was to mix in a series of flasks, 150 cc. of stock casein solution with the indicated amount of trypsinogen in solution and water to make up to 180 cc. At proper time intervals, 20 cc. of the indicated enterokinase solution were added and the time of digestion calculated from the time of this addition. The values of R. I. Δ were corrected by graphical interpolation from the standard curve, when the change was more than 4.5 scale divisions. This correction was made in order to correct for the deviation of the casein from the simple unimolecular Equation 6 that was combined with Equation 8 to get Equation 9, which is used to interpret the data. The correction was made on the assumption that the deviation from the unimolecular rate of reaction is entirely a function of the amount digested.

The experimental values for K, T, t^2, and R. I. Δ were substituted in Equation 9 and the constant k_4 calculated. In all diges-

TABLE I

Activation in Presence of Substrate with Variable Trypsinogen

The values in parentheses are the values corrected graphically.

Mg. per 200 cc.		Digestion		k_4	Mg. per 200 cc.		Digestion		k_4
T_7	K_3	Time	Amount R. I. Δ		T_7	K_3	Time	Amount R. I. Δ	
		hrs.					*hrs.*		
50	0.48	0.5	0.9	0.150	6	0.2	1.0	0.55	0.458
		0.75	1.85	0.137			1.5	1.3	0.480
		1.0	3.1	0.129			2.0	2.4	0.500
		1.25	4.5	0.120			3.0	4.95 (5.2)	0.460
		1.50	5.7 (6.41)	0.118			4.0	7.00 (8.7)	0.455
		2.0	7.8 (10.2)	0.106	4	0.5	0.75	0.55	0.487
30	0.48	0.5	0.7	0.194			1.0	1.2	0.600
		0.75	1.55	0.191			1.25	1.7	0.542
		1.0	2.7	0.187			1.5	2.5	0.555
		1.25	4.1	0.182			2.0	4.3	0.537
		1.5	5.4 (5.9)	0.182			3.0	6.9 (8.6)	0.477
		2.0	7.4 (9.8)	0.170	2	0.5	1.0	0.65	0.650
20	0.2	0.5	0.3	0.300			1.5	1.5	0.665
		0.75	0.70	0.310			2.0	2.6	0.650
		1.0	1.2	0.300			3.0	4.9 (5.2)	0.575
		1.25	2.0	0.320			4.0	6.9 (8.6)	0.535
		1.5	2.8	0.310	1.2	0.5	1.0	0.5	0.833
		2.0	4.6	0.288			2.0	1.7	0.708
		3.0	7.45 (9.9)	0.275			3.0	3.3	0.610
16	0.2	0.75	0.65	0.359			4.0	5.2 (5.6)	0.583
		1.0	1.05	0.329			5.0	6.7 (8.2)	0.547
		1.25	1.7	0.338	0.8	0.5	1.0	0.25	0.625
		1.5	2.35	0.325			2.0	1.15	0.717
		2.0	4.1	0.318			3.0	2.25	0.625
		3.0	6.9 (8.5)	0.318			4.0	3.8	0.592
12	0.2	0.75	0.55	0.406			5.0	5.25 (5.7)	0.570
		1.0	0.9	0.375			6.0	6.5 (7.7)	0.533
		1.25	1.5	0.400	0.4	0.5	2.0	0.65	0.810
		1.5	2.15	0.398			3.0	1.1	0.610
		2.0	3.7	0.385			4.0	2.0	0.625
		3.0	6.4 (7.7)	0.356			5.0	2.9	0.580
		4.0	8.8 (13.8)	0.359			6.0	4.1	0.565
8	0.2	0.75	0.35	0.389	10		2.0	0.0	
		1.0	0.65	0.406			4.0	0.0	
		1.25	1.1	0.406			6.0	0.0	
		1.5	1.65	0.457			8.0	0.0	
		2.0	3.05	0.476			10.0	0.1	
		3.0	5.8 (6.5)	0.451			24.0	0.3	
		4.0	7.85 (10.8)	0.421					

tion mixtures, the value of this constant decreased with time. This drift is a function of time and seems to be almost independent

TABLE II

Activation in Presence of Substrate with Variable Enterokinase

The values in parentheses are the values corrected graphically.

Mg. per 200 cc.		Digestion		k_4	Mg. per 200 cc.		Digestion		k_4
T_7	K_3	Time	Amount R. I. Δ		T_7	K_3	Time	Amount R. I. Δ	
		hrs.					*hrs.*		
4.0	0.2	1.0	0.55	0.688	8.0	0.08	1.0	0.35	0.546
		2.0	2.35	0.733			1.5	0.85	0.589
		3.0	4.65	0.645			2.0	1.50	0.585
		4.0	6.3 (7.5)	0.585			3.0	3.20	0.554
		6.0	8.5 (13.1)	0.453			4.0	5.05 (5.4)	0.526
		9.0	11.0 (24.5)	0.378			6.0	7.55 (10.2)	0.441
4.0	0.08	2.0	1.0	0.780	8.0	0.04	1.5	0.25	0.346
		3.0	1.95	0.675			2.0	0.80	0.625
		4.0	3.2	0.625			3.0	1.65	0.571
		6.0	5.8 (6.5)	0.563			4.0	2.90	0.565
		9.0	8.4 (12.6)	0.485			6.0	5.05 (5.40)	0.469
4.0	0.06	2.0	0.7	0.728			8.0	7.0 (9.0)	0.438
		3.0	1.35	0.625	8.0	0.02	2.0	0.40	0.625
		4.0	2.3	0.595			3.0	0.85	0.588
		6.0	4.7	0.540			4.0	1.50	0.581
		9.0	7.35 (9.7)	0.495			6.0	2.85	0.494
4.0	0.04	2.0	0.5	0.780			8.0	4.6	0.444
		3.0	0.85	0.595			10.0	6.0 (6.8)	0.425
		4.0	1.65	0.640	8.0	0.01	3.0	0.45	0.625
		6.0	3.4	0.595			4.0	0.75	0.575
		9.0	5.8 (6.5)	0.500			6.0	1.45	0.500
4.0	0.02	2.0	0.2	0.625			8.0	2.5	0.488
		3.0	0.4	0.550			10.0	3.5	0.438
		4.0	0.75	0.575			23.0	8.6 (13.5)	0.313
		6.0	1.65	0.563	8.0	0.006	4.0	0.45	0.583
		9.0	3.5	0.540			6.0	0.85	0.479
4.0	0.01	2.0	0.1	0.625			8.0	1.50	0.479
		3.0	0.25	0.675			10.0	2.20	0.458
		4.0	0.35	0.550			23.0	6.5 (7.8)	0.291
		6.0	0.8	0.550					
		9.0	1.7	0.500					

of the concentration of the trypsinogen and enterokinase or the amount of digestion. Anything that will decrease the trypsin

content during the reaction will decrease the value of the constant k_4. Such factors are thermal destruction of trypsinogen, enterokinase, and trypsin, destruction of the same components by the action of trypsin, decrease in the quantity of trypsinogen by conversion into trypsin, which has been assumed to be negligible in the derivation of Equation 9, and combination of any of the three components with other constituents of the reaction mixture.

When the amount of enterokinase was maintained constant (see Table I) the amount of digestion was not directly proportional to the trypsinogen when relatively high concentrations of it were present. When small concentrations of trypsinogen were used, the amount of digestion was, however, more nearly proportional to the amount of trypsinogen. The lower values of k_4 at high concentrations of trypsinogen are associated with an increasing rate of digestion which may involve a limiting factor such as intermediate compound formation that is not accounted for in the equation. This decrease in k_4 is not due to the increased ratio of T_7 to K_3 as can be seen from the values in the last mixture in Table II, where the ratio was 1330:1.

When the amount of trypsinogen was constant (Table II) the amount of digestion was directly proportional to the amount of enterokinase, provided an excess of trypsinogen was present, or that the reaction was not considered after much of the trypsinogen had been activated as in the maximal activation discussed previously. This proportionality, as shown by the values of k_4, holds only for any constant time of digestion since the value of k_4 decreases with time.

We previously showed that 0.1 mg. of K_1 was capable of activating, in the absence of substrate, only 1 unit of trypsinogen according to the addition compound theory. Since K_3 is 4 times more potent than K_1, we calculate that 0.006 mg. of K_3 is equivalent to 0.024 mg. of K_1, which should be able to activate only 0.24 unit of trypsinogen. In the last mixture in Table II, the amount of digestion between the 8 and 10 hour samples was equivalent to that obtained from 1.4 units of trypsin, which is 6 times the quantity allowed by the addition compound theory. A similar calculation on the 8 to 10 hour interval with 8.0 mg. of T_7 and 0.01 mg. of K_3 shows the amount of digestion obtained to be 5 times that permitted by this theory. More confirmatory values can be

obtained from the other mixture at similar times. The amount of trypsin in the solution, *i.e.* the rate of digestion, increases still further after 10 hours.

Quantitative Assay of Enterokinase—This finding of a constant value for k_4 suggests an exact and accurate method for the comparative assay of enterokinase, which is almost as accurate as the method for the assay of trypsin. It was used to get the relative potencies of the kinase preparation described. For the assay of enterokinase, one should choose a quantity of trypsinogen equivalent to 100 units and a preparation of trypsinogen so free from trypsin that 2 to 5 times the quantity used will give no digestion in 4 hours with the substrate in the absence of any kinase. This constant amount of trypsinogen is mixed with the substrate and varying quantities of a standard enterokinase preparation and allowed to act for a definite period of time (4 hours). The amount of digestion is then determined and plotted against the quantity of enterokinase. A straight line will result if the digestion values are corrected by interpolating from the standard curve. The slope of this line is the product of $(k_4 \times T \times t^2)$, the absolute value being dependent upon the units of measure chosen. Unknown enterokinase preparations can be assayed with one or more different concentrations of enterokinase, all other factors being identical with those used in making the standard enterokinase curve. The potency of the unknown is read directly from the standard enterokinase curve in terms of the enterokinase preparation chosen as a standard. The determinations are most accurate when the R. I. Δ is between 3 and 5 scale divisions. The quantity of enterokinase which gives most accurate results by this method is less than 0.1 the quantity needed to obtain an accurate assay by the maximum activation method.

Stability Studies

Many reports on the stability of trypsinogen, enterokinase, and trypsin are to be found in the literature. Recently, Pace (8) reported trypsinogen, enterokinase, and trypsin to have a maximum stability around pH 6.0 and 6.5. Northrop and Kunitz (9) found trypsin most stable around pH 5.0, but crystalline trypsin in the presence of 0.25 saturated ammonium sulfate had a maximum stability at pH 2.0.

The method of assaying used to obtain the data in Table III can be understood better after consideration of Fig. 6. The quantity of K_3 used was sufficient to give a result equivalent to about 0.9 complete activation. Table III shows that, contrary to the finding of Pace, the maximum stability of trypsinogen is between pH 2.0 and 3.0. It is less stable in alkaline solution. It is also rapidly destroyed when the pH is less than 1.0. Enterokinase is rapidly

TABLE III

Stability of Trypsinogen at 37.5°

After standing at the pH and for the time indicated, 10 cc. samples were added to a mixture of 75 cc. of casein + 15 cc. of kinase (1 mg. of K_3) for 4 hours. All values are scale divisions of the refractometer.

Time	Portions contained 0.3 mg. of T_7							0.5 mg. of T_2		
	pH 1.37	pH 2.08	pH 2.35	pH 3.35	pH 4.70	pH 6.1	pH 6.1, 0.05 N KCl	pH 10.0	pH 11.7	pH 11.7, 0.05 N KCl
0 hr.	5.5			5.65		5.7	5.6	7.6	7.6	7.55
2 hrs.								8.1	6.6	4.60
3 "	5.5	5.55	5.55	5.45	5.45	5.6	5.35	7.65	3.35	0.90
24 "	4.8	5.15	5.05		5.2	5.2	5.6	1.05	0.3	0.3
49 "	5.5	6.2	6.4	6.3	6.4	3.0	5.5			
100 "	4.5	5.6	5.8	5.8	0.6	0.2	4.0			
170 "	3.2	5.0	5.2				1.0			
30 min.*	0.25	1.05	2.10	5.75	5.6	5.9	6.05			

* Activated in the absence of the casein substrate with the same large excess of kinase for 30 minutes at 37.5° and the resulting pH (add 0.3 pH except at pH 6.1). The results are a measure of the destruction of enterokinase.

destroyed (1 hour at 37.5°) in both 0.01 N acid and alkaline solution (see also Table III and Fig. 4).

The unimolecular constant, $k_{uni.}$ (time in minutes) for the thermal destruction of trypsin at 37.5° and pH 6.0 in the almost complete absence of electrolyte was 2.1×10^{-3} (90 per cent destroyed in 24 hours). In the presence of 0.05 N KCl at the same temperature and pH, $k_{uni.}$ was 1.0×10^{-3}; *i.e.*, the stability was doubled. On the other hand, 0.05 N KCl increases the rate of destruction of trypsinogen at pH 11.7.

DISCUSSION

This comparative study of the activation of trypsinogen with enterokinase in the absence and the presence of the casein substrate has yielded results which are at variance with the addition compound theory. The addition compound theory, as expressed by Equation 2, requires that trypsinogen and enterokinase play parts of similar or equal importance in the formation of trypsin. If trypsinogen and enterokinase were of equal quantitative significance, Figs. 2 and 3 should be similar. They are not. The differences between the results shown in Figs. 2 and 3 are readily explained, however, by assuming that enterokinase acts enzymatically. Also, in the presence of the casein substrate, a great excess of enterokinase causes complete activation of the trypsinogen within a few minutes. The maximum ratio by weight of $T_2:K_1$ (Fig. 6) was 1:20, which is equivalent to a ratio of 1:5 for $T_7:K_3$. On the other hand, a great excess of trypsinogen does not cause a rapid and complete stoichiometric combination with all of the enterokinase to give the hypothetical trypsinogen-enterokinase complex and which would cause digestion at a rate proportional to the first power of the time. What does happen is activation at a slow constant rate so that the amount of trypsin, as measured by casein digestion, increases with time. Thus, in the last experiment (Table II) the ratio of $T_7:K_3$ is 1330:1 and in spite of this great excess of trypsinogen there is no indication of rapid conversion of all the kinase into the trypsinogen-enterokinase complex. Instead, the amount of digestion is proportional to the square of the time, as is expected, if enterokinase acts as an enzyme.

Kunitz and Northrop (10) have recently shown that the activation of trypsinogen will occur autocatalytically under certain conditions. None of our activation experiments gave evidence of an autocatalytic reaction.

We do not doubt that there occurs a transient combination between molecules of trypsinogen and enterokinase during the activation process, but we believe that our data disprove that this combination persists and that it is the proteolytically active enzyme.

SUMMARY

1. A simple method for the preparation of trypsinogen, free from trypsin and enterokinase, is described.

2. Trypsinogen is more stable at pH 2.5 than at pH 6.0. Low concentrations of KCl (0.05 N) stabilize trypsinogen or trypsin in neutral solutions. Enterokinase is rapidly destroyed in solutions at pH 2.5 and 11.7.

3. The effect of pH and concentration of KCl on the rate of activation of trypsinogen by enterokinase is shown.

4. The effect of varying the ratio of trypsinogen to enterokinase on the rate of activation of trypsinogen in the absence of a substrate and the maximum yield of trypsin resulting are studied.

5. It is shown that activation of trypsinogen by enterokinase occurs in the presence of the substrate at a pH of 8.2. The resulting digestion, expressed in terms of change of refractive index in scale divisions of a dipping refractometer between the isoelectric filtrates of the casein substrate before and after digestion, is represented approximately, by the equation R. I. $\Delta = k \times T \times K \times t^2$, where T is trypsinogen, K is enterokinase, t is the time of digestion, and k is a constant.

6. A simple accurate method for the assay of enterokinase is described.

7. The results show that enterokinase acts as an enzyme in the activation of trypsinogen.

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